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Appn. Filed: 12/05/2001
Appn. #: 10/679,305
Applicant: Elizabeth Gay Frayne
Title: "Manipulation of RNA Stability and Protein Synthesis Using Thiophosphate"
Examiner: Nancy T. Vogel
Art Unit:1636

Assistant Commissioner for Patents
Arlington, Virginia, 22313

RE: Office Action 3/1/11

Below is my response to the examiner's comments and objections in her last office action. I would like to segregate claim 9 into a new continuation in part application with a new title because it is not fully explained in the present application. I have modified it in the present response to show the direction I am going with it but upon reflection I see that I have not adequately described how to detect the modification for the applications intended.

As claim 8 was previously accepted, I hope this issue can be resolved, see comments below. I present my arguments in favor of claim 10 which I hope overcome the examiner's objections. I have also modified the claim to make it more clear. I hope to keep both claim 8 and 10 in the present application.

Claim 8

Claim 8 had previously been accepted using the word micro-organisms. In the last amendment cell culture was used instead. The examiner did not state that the modified claim was accepted but also did not provide any comments specifically addressing this claim in her response. Please modify this claim as appropriate to make it acceptable.

Claim 9

thio-phosphate to phosphate ~~containing culture media~~

2) testing the collection of different medias to determine ~~determining~~ the optimal ratio of thio-phosphate to ~~inorganic~~ phosphate in the culture media for maximum induction of protein synthesis from a marker gene whose mRNA falls within the class of those mRNAs with half-lives shorter than average ~~in the culture media that enables the desired mRNAs to preferentially accumulate and/orcompete for translation.~~

3) further maximizing the induction of protein synthesis from the said marker gene by determining the optimal time point for ~~to induce stabilization of the desired mRNAs via~~ the addition of thio-phosphate containing media to the culture of micro-organisms ~~cultures that results in maximal protein synthesis of the corresponding gene products~~

11. (previously amended)

The method of claim 10 wherein the cells used to enhance the stability of the mRNAs and their respective proteins are prokaryotic or eukaryotic in origin.

12. (currently amended)

The method of claim 10 wherein the mRNAs preferentially stabilized are produced ~~derived~~ from a recombinant DNA or viral expression vector or are produced by an intact virus.

13. (previously amended)

The method of claim 10 wherein the accumulation of the stabilized mRNAs and their respective proteins results in an increase in a rate limiting enzyme(s) needed for the synthesis of a given organic chemical fermentation product.

14. (cancelled)

Claim 9 addresses the use of the modified phosphate as a labeling method for nucleic acids insides cells. This claim is less stringent since it does not imply altering the stability of the RNA or DNA significantly but rather simply modifying it enough for detection purposes. In the first paragraph of the Description of the Preferred Embodiments I address the utility of being able to label cells for the purpose of detecting viable cells (growing or resting) as opposed to dead cells. I address the potential use of thio-phosphate for imaging purposes with the possibility of using NMR for in vivo detection of the modified phosphate. This clearly identifies utility in being able to use thio-phosphate as a ***non-radioactive*** labeling reagent. A labeling reagent only requires enough material for detection purposes and in this case does not require complete substitution.

I have demonstrated in bacterial cells and yeast cells that you can fully substitute the RNA and/or DNA with modified phosphate, so clearly it should be possible to do so in the more limited case, namely, for detection purposes. For other eukaryotic cells one could imagine injecting a bolus directly into the blood or other body fluids allowing for direct contact of the solution with the cell. Direct contact of cells in vivo with the modified phosphate should also allow for its uptake even in the presence of normal phosphate levels as long as an excess of thio-phosphate is used. Thio-phosphate is incorporated as readily as normal phosphate so even a mixture of 50% thio-phosphate to normal phosphate would allow significant incorporation of thio-phosphate. This was evident in examples using different ratios of thio-phosphate to phosphate showing incorporation into nucleic acids and with subsequent effects on protein synthesis.

In the case of the gold fish the liquid in the aquarium containing thio-phosphate was taken up into the gut (a hostile environment) where it was diluted (but potentially equilibrated), and successfully competed with endogenous sources present. The 250 fold excess refers to the amount of thio-phosphate added relative to the amount present in the aquarium with feed (gold fish flakes). The concentration of phosphate in fish blood (Voitsitsky et al, 2006)) is much higher in the mM range similar to what was used in the modified fish aquarium media based on molecular weight. So in that sense the thio-phosphate was not in great excess but still clearly able to compete with endogenous sources. This is analogous to injecting thio-phosphate into blood and exposing cells in vivo to this compound for uptake and incorporation into nucleic acids.

I have modified claim to indicate uptake of thio-phosphate into cells upon direct contact with liquid media or bolus. A bolus in this sense refers to the administration of a compound that is given to raise its concentration in the blood or interstitial fluid to an effective level. I have shown that upon direct contact of cells from a variety of organisms such as bacteria, yeast, and fish intestinal cells thio-phosphate is taken up by cells into nucleotide precursor pools and can modify nucleic acids.

Claim 10

Examiner argues that the assertion that some mRNAs can be preferentially enhanced over others is mere speculation. This argument is not rationale. The use of thio-phosphate clearly can be used to enhance or protect an mRNA from degradation via the formation of phosphorothioate linkages. Based on the inherent variability of mRNAs it is ***a priori*** possible to predict that shorter lived mRNAs can be preferentially enhanced following uptake prior to reaching equilibrium. At equilibrium if all mRNAs are enhanced equally by say complete substitution with thio-phosphate then there will be no difference in the stability of these mRNAs. However, in the approach to equilibrium or steady state, the shortest lived mRNAs will be preferentially enhanced because they turn over faster enabling a greater degree of substitution sooner than in mRNAs that are longer lived. With less than complete substitution of the mRNA backbone, again one has the same prediction. There is also the possibility that a more modest degree of substitution in a more stable mRNA species will have no effect while in is a less stable mRNA species the modification will be of great benefit. This could be influenced by things like the secondary structure of the mRNA or the binding of proteins (see E. coli example below).

When first developing methods for enhancing protein synthesis an obvious question was does simply adding thio-phosphate increase total soluble protein synthesis? It was found (see experimental section 2 last paragraph) that at concentrations that do enhance protein synthesis of measured proteins significantly there is no increase in total soluble protein synthesis. Furthermore, at very high ratios of thio-phosphate to phosphate in the media there is a slight reduction in total protein synthesis as opposed to an increase that appears to be accounted for by a slight reduction in cell growth. This implies that for enhanced protein synthesis of measured proteins, that as predicted some mRNAs preferentially accumulate over others leading to their enhanced translation. The mechanism for this is as discussed above based on preferential enhancement of the stability of less stable mRNAs. It also follows that if total protein synthesis is not increased and instead stays the same instead, then some mRNAs must be translated less readily, namely, those that are more stable as their accumulation is not enhanced. A good example of this was for beta-galactosidase in E. coli (paragraph 6 experimental section second example) which was tested under inductive and non-inductive conditions. The enzyme activity was actually inhibited using thio-phosphate under inductive conditions where it would be expected to accumulate. In contrast, it was induced under non-inductive conditions where it would be expected to be unstable due to a deficiency of protective ribosomes used for protein translation.

In a number of systems and for a number of different genes it was possible to enhance the protein synthesis levels of measured genes using thio-phosphate. The common thread in these examples is enhanced stability of mRNA species in cells owing to the substitution of their nucleic acid backbones with phosphorothioate linkages. Such modifications are known to enhance the stability of nucleic acids in vivo. The present invention provides a way to manipulate the stability of nucleic acids generated in vivo using this same principle. It is not speculative and it is substantiated by the findings.

The specification provides adequate instruction for the use of the method. At least two other related patents have issued pertaining to the use of thio-phosphate in culture media. Note the parent application is incorporated by reference. The only difference in the present application is its use to enhance protein synthesis. Rather than using media that is completely substituted with thio-phosphate, the optimal amount of protein synthesis occurs at a specific ratio of thio-phosphate to normal phosphate in the media. The idea of a ratio is important as thio-phosphate competes with normal phosphate when present in the media. The specification gives several examples showing the effects of different ratios on RNA accumulation and stability. There are two approaches to preparing media described in the experimental section. One can prepare chemically defined media and substitute the phosphate with thio-phosphate. Different ratios can be created by diluting such media with normal media. A 50:50 mixture would create a 50:50 ratio. Another approach is to use nutrient broths depleted of endogenous phosphate by precipitation and then adding back thio-phosphate or phosphate and preparing different ratios of these. The ratios used are based on weight equivalent rather than molar equivalent. Better growth is obtained with nutrient broths as opposed to minimal medias in bacteria or yeast but both types of medias result in induction at similar ratios of thio-phoshate to phosphate. The experimental section (fourth paragraph of section two) also describes simply adding thio-phosphate to nutrient broths to obtain ratios less than 100% thio-phosphate needed for induction. It describes measuring the amount of phosphate in nutrient broths and comparing it with a standard salt solution of phosphate to determine the appropriate amount of thio-phosphate to add based on weight. There is nothing complicated about preparing media with different ratios of thio-phopshate to phosphate.

The next step of claim three deals with the timing of the addition of thio-phosphate. Obviously thio-phosphate must be present in the media prior to mRNA synthesis of the desired gene products for the method to work. Many genes are activated during growth (trophic phase) while others are activated when cells are in the stationary phase (idiophase) (See the Description of Preferred Embodiments paragraphs 5 & 6). The experimental section provided examples of induction via thio-phosphate in both situations. Induction was seen during growth in bacterial cells for the AP and Beta-galactosidase genes. The yeast recombinant system tested the BAP gene under control of the ADH promoter which is not actually activated until glucose levels are exhausted. This represents gene activation as cell are slowing down and entering the resting phase due to glucose depletion in the media. The additional native secreted proteins in yeast examined appeared to have similar kinetics to the ADH regulated recombinant gene.

In any event it is common to optimize protein expression in systems by varying the time of growth for maximum expression. The third claim simply states that by varying the timing of thio-phosphate addition to cultures you may optimize the accumulation and expression of the desired mRNAs and their corresponding gene products. The examples in the experimental section all involved dilution of cells into thio-phosphate containing media at the beginning of the growth phase. This was very convenient to do in these short lived culture conditions. For situations that require longer periods of culture, the timing of the addition of thio-phoshate may be helpful for optimizing the system. It was indicated that thio-phosphate does breakdown particularly under acidic conditions and so for longer culture conditions you may want to add it closer to when it is being used for mRNA synthesis (see experimental section example 2 paragraph 4).

I believe there is plenty of evidence to support the steps of claim 10 in the specification as it speaks to the heart of the invention. It is not a new idea. Below is a quote from the original parent application "Cells grown in 100% thio-phosphate substituted media generate higher yields of mRNA per cell. At lower ratios of thio-phosphate to phosphate many cellular mRNAs are preferentially stabilized over more abundant species resulting in a concomitant increase in the protein synthesis of such mRNAs. The novel mRNA stabilizing media can be used to increase the protein levels of native and/or recombinant proteins in prokaryotes as well as eukaryotes." The present application further suggests uses for secondary consequences of enhanced mRNA expression and protein synthesis in cells. Namely, if rate limiting enzymes or proteins are enhanced in cells then processes dependent on these proteins may also be enhanced.

Reference:

1. Voitsitsky, V. et al (2006) Macro-and microelemental composition of the antartic fish blood serum. *Ukrainian Anartic Journal*. Vol. 4-5, pgs 246-250. Retrieved from http://www.uac.gov.ua/science/journal4_5/UAJ_N_4-5_246-250.doc

I wish to declare and make a verified statement that the modified claims include no new matter as required by 37 CFR 1.821(e), 1.821(f), 1.821(g), 1.825(b) or 1.825 (d).

Respectfully submitted,

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